NUCLEOLAR DNA IN OOCYTES OF
XENOPUS LAEVIS

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SUMMARY

The ovaries of newly metamorphosed Xenopus females contain oocytes in all stages of early meiotic prophase. In pachytene nuclei extrachromosomal nucleolar DNA appears in the form of a thin cap covering one side of the nucleus. During pachytene this cap of DNA enlarges to occupy half the nucleus. After pachytene the nucleus grows rapidly and the cap of DNA disperses into numerous tiny granules which become scattered throughout the nucleoplasm. Autoradiographs of cells which have been incubated with $^3H$thymidine, and microspectrophotometric measurements of the Feulgen dye contents of nuclei in various stages of meiosis, show that the extrachromosomal nucleolar DNA is synthesized during the pachytene and immediate post-pachytene stages. Microspectrophotometric comparison of the Feulgen dye contents of post-pachytene nuclei in which the DNA cap has dispersed, and similarly prepared mouse liver nuclei, show that the post-pachytene nuclei have about 30 μg of extrachromosomal pachytene nucleolar DNA. In $^3H$thymidine autoradiographs early pachytene nuclei are less heavily labelled than late pachytene and early diplotene nuclei. Consequently it is proposed that primary replicas of the chromosomal nucleolar organizer undergo a series of post-detachment replications.

INTRODUCTION

An amphibian oocyte has many nucleoli. These are homologous with the nucleoli of somatic cells so far as their genetic origin is concerned (Gall, 1954; Callan, 1966). They are also uniform with respect to their function in so far as they are all actively engaged in the synthesis of RNA having a base composition similar to that of cytoplasmic ribosomal RNA (Edström & Gall, 1963; Macgregor, 1967). Each nucleolus contains a small amount of DNA (Miller, 1964), and it has been suggested that this nucleolar DNA arises early in oogenesis through a multi-replication of the chromosomal nucleolar organizer (Miller, 1964; Macgregor, 1965; Miller, 1966). Two sets of observations support this suggestion. First, Painter & Taylor (1942) showed that in oocytes of Bufo valliceps extrachromosomal DNA appears during pachytene as numerous small Feulgen-positive granules. These granules move to the periphery of the nucleus. Later in oogenesis each granule has one nucleolus associated with it, the granule lying between the nucleolus and the nuclear envelope. Secondly, Gall (1967) has shown that in ovaries of newly metamorphosed Xenopus, in which most of the oocytes are in early prophase of the first meiotic division, there is a disproportionately intense synthesis of a fraction of DNA with a buoyant density greater than is usual for Xenopus DNA. Gall suggests that this fraction is heavy on account of a high G+C content and that it is nucleolar DNA which codes for ribosomal RNA.
Much is known about the nucleolar organizer of *Xenopus* from the work of Birnstiel, Wallace, Sirlin & Fischberg (1966) and Wallace & Birnstiel (1966) on the location of ribosomal cistrons in the nucleolar organizer, and from Brown & Gurdon's (1964) observations on the absence of ribosomal RNA synthesis in anucleolate mutants of *Xenopus*. I therefore chose to examine the production of extrachromosomal nucleolar DNA in *Xenopus* and to measure the amount of nucleolar DNA per oocyte nucleus. The observations come from squash preparations of *Xenopus* ovaries stained with the Feulgen technique, and from [H]thymidine autoradiographs, and they include microspectrophotometric measurements of the Feulgen dye contents of oocyte nuclei.

**MATERIALS AND METHODS**

Female *Xenopus* weighing between 0.4 and 1.0 g were used as a source of material for all observations. The animals were generously supplied by Dr M. L. Birnstiel of the Institute of Animal Genetics, Edinburgh.

Freshly excised ovaries, each no larger than a pin-head, were fixed for 2 h in ethanol-acetic acid (3:1) (Clarke, 1851). They were then placed in 45% acetic acid at 22 °C for 30 min, after which pieces of tissue were tapped out on microscope slides to dissociate the cells, covered with coverglasses, and squashed lightly by laying the slide, coverglass downwards, on a clean filter paper. One ovary provided 3 or 4 squash preparations. The coverglasses were removed by the 'dry-ice' method. The preparations were hydrated, hydrolysed by immersion in N HCl at 60 °C for 11 min, stained in Feulgen's reagent (Swift, 1955) for 2 h, washed in SO₂ water (Swift, 1955), 3 changes of 10 min each, dehydrated in ethanol, cleared in xylene, and mounted in immersion oil (nD 1.515).

For autoradiography freshly excised ovaries were incubated for 6 h in a sterile medium which consisted of 10 ml tissue culture medium TC 199 (Difco Laboratories, Inc., Detroit, U.S.A.), 6 ml water, 0.03 g/ml crystalline bovine plasma albumin (Armour Pharmaceutical Co., Ltd., Eastbourne, England), and 50 μl (50 μc)/ml of a solution of [H]thymidine (1.9 c/mm) supplied by The Radiochemical Centre, Amersham, England. After incubation the ovaries were fixed in ethanol-acetic acid (3:1) and squashed as described above. Squash preparations were hydrated, immersed in 5% trichloroacetic (TCA) at 5 °C for 10 min with constant agitation, washed in water, dehydrated in ethanol and acetone, and air-dried. Dry preparations were coated at 45 °C with Kodak NTB2 liquid emulsion diluted 1:1 with water. They were then placed in light-tight boxes and left to expose for 7 days at 22 °C. Autoradiographs were developed in Kodak D 19b for 3 min, washed in water, fixed for 5 min in Kodak acid fixer, and washed in running water. They were then stained for 10 min in 0.15% methyl green (pH 6), washed in buffer, dehydrated in ethanol, cleared in xylene and mounted in balsam.

Measurements of the Feulgen dye contents of nuclei were made with a Carl Zeiss photomicroscope fitted with a Carl Zeiss 'spectrophotometer for microscopes'. The latter equipment consists of a stabilized low-voltage illuminator, a 50 c/s modulator, an interference graduation filter monochromator, a 0.4 mm aperture interposed...
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immediately above the monochromator between it and the condenser, an achromatic aplanatic condenser stopped down to a numerical aperture of about 0.3, a 10 × planapochromatic objective (N.A. 0.32), a 10 × or 20 × projector lens, a 5 mm aperture, a photomultiplier, and an indicator system. With the 10 × projector lens the measured area is a circle of radius 20 μ; with the 20 × projector the measured area is a circle of radius 10 μ. Feulgen dye contents of nuclei were determined by the 2-wavelength method of Patau (1952). The wavelengths chosen for each preparation were such that for an apparently homogeneous nucleus filling and overlapping the boundary of the measured area the extinction at the lower wavelength was half that at the higher wavelength. The higher wavelength was 570 μ for all preparations. The lower wavelength was varied slightly from preparation to preparation and ranged from 507 to 512 μ. Since some measurements which were to be compared with one another were made with the 10 × projector and some with 20 × projector the transmissions of these two lenses at the measuring wavelengths were carefully checked. The lenses were found to be precisely matched.

The Feulgen dye contents of nuclei were expressed as \((1 - T_1)C_A\), where \(T_1\) is the transmission at the lower wavelength, \(C\) is obtained from \((1 - T_1)/(1 - T_2)\) using Patau’s (1952) tables, and \(A\) is the measured area in μ².

All measurements were made on whole nuclei in Feulgen-stained squashes prepared as described above. All preparations received precisely the same treatment with respect to every step in the procedure from fixation to final mounting.

In an early series of measurements an attempt was made to compare the Feulgen dye content of oocyte nuclei with that of erythrocyte nuclei and follicle epithelium nuclei in the same preparation. For a variety of reasons, however, both erythrocyte and follicle cell nuclei proved unsatisfactory as standards, and therefore a more reliable standard was sought. The procedure adopted was to fix, squash, stain, and mount a sample of mouse liver alongside each ovarian sample. The mouse liver was chopped as finely as possible with a razor blade and underwent precisely the same fixation and squashing procedure as the *Xenopus* ovary and, since liver and ovary were squashed on the same slide, both underwent identical hydrolysis, staining, and mounting procedures. Mouse liver proved particularly suitable since the octoploid nuclei, when slightly compressed, were of about the same size and shape as the larger oocyte nuclei in the *Xenopus* squashes. The Feulgen dye contents of both types of nucleus could therefore be measured under the same optical conditions.

**OBSERVATIONS**

The nuclei of oogonia are irregular in shape and measure about 12 μ across (Fig. 1). The chromatin is evenly distributed throughout the nucleus in the form of a fine reticulum. In early leptotene and zygotene the nuclei are about 10 μ in diameter and the chromatin is more compact (Fig. 2). During pachytene a cap of Feulgen-positive material forms over one side of the nucleus (Fig. 3). In pachytene nuclei the chromosomes are arranged in an untidy bouquet. The cap of Feulgen-positive material is much enlarged. It occupies about half the nucleus, and at this stage, stains intensely.
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(Figs. 4, 5). The ends of the chromosomes point away from the cap. After pachytene the nuclei enter a diffuse diplotene stage from which the germinal vesicle and its lampbrush chromosomes develop directly. The nuclei enlarge progressively. There is a concurrent elongation of the chromosomes and disappearance of the bouquet arrangement. As the nucleus enlarges the chromatin cap becomes more diffuse and granular in appearance (Figs. 5-7). By the time the nucleus reaches a diameter of about 25 µ the chromatin cap has completely dispersed and larger nuclei are uniformly granular in appearance (Figs. 8, 9).

In autoradiographs prepared as described above zygotene nuclei were unlabelled. All nuclei in the cap stages of meiotic prophase had a high concentration of silver grains over the cap, whereas the chromosomes were unlabelled (Figs. 11, 12). Nuclei over 20 µ in diameter in which the cap had partially or completely dispersed were unlabelled (Fig. 13).

Nuclei in different stages of cap formation were labelled to different degrees. In early pachytene nuclei 20–50 silver grains were clustered around the edges of each nucleus (Fig. 10). In later pachytene nuclei 100–150 silver grains overlay each cap (Fig. 11). In late pachytene nuclei with large caps 150–200 silver grains were concentrated over each cap (Fig. 12).

Table 1. *Feulgen dye content values of oocyte nuclei and octoploid mouse liver cell nuclei*

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Feulgen dye content values are expressed in arbitrary units. Each column represents measurements from a different preparation and animal.

Feulgen dye measurements of oocyte nuclei were made with three objectives in mind. The first was to ascertain that oocyte nuclei contain more than the 4C quantity of DNA usual for *Xenopus*. The second was to determine when the DNA content of
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Oocyte nuclei first exceed the 4C value for *Xenopus*, and when it reaches a final maximum value. The third objective was to obtain some measurement of that final value.

Feulgen dye content (FDC) measurements were made on pre-pachytene nuclei, on early and late 'cap' nuclei, on post-cap nuclei in which the cap formed a diffuse mass occupying half the nucleus, and on the largest measurable oocyte nuclei. The latter were 25-30 μ in diameter in squash preparations and were more or less homogeneous in appearance. Nuclei were considered unmeasurable if the transmission through them of light of the lower of the two wavelengths used exceeded 90%. A typical series of measurements, together with photographs of the measured nuclei, is shown in Figs. 14-21. These and other similar measurements showed that in pre-pachytene nuclei the amount of DNA present approximates to the 4C value for *Xenopus*, that there is a rapid build-up of nuclear DNA during pachytene and the cap stages, and that an apparent maximum DNA value is reached at diffuse diplotene soon after the cap disperses.

Five sets of measurements were made on post-cap nuclei of more than 25 μ diameter in 5 preparations, each of which came from a different animal. Five oocyte nuclei and five mouse liver nuclei (octoploid) were measured in each preparation. The results are shown in Table 1.

The 2C value for mouse is about 6·5 μg DNA. The amount of oocyte nuclear DNA for each of the animals sampled is therefore: (1) 43 μg, (2) 42 μg, (3) 41 μg, (4) 40 μg, and (5) 47 μg. The overall mean amount of oocyte nuclear DNA is therefore 42·6 μg. Subtracting from this the 4C value for *Xenopus*, 12 μg (Wallace & Birnstiel, 1966), 30·6 μg of nucleolar DNA remain.

**Discussion**

The observations presented in this paper prove the existence of relatively large amounts of extrachromosomal DNA in young *Xenopus* oocytes. Because of the findings of Painter & Taylor (1942), Miller (1964), and Gall (1967) one may suppose that most, if not all, of this DNA is nucleolar. The synthesis of nucleolar DNA begins in leptotene nuclei. An estimate has been given of the amount of DNA present in each oocyte, but before this estimate can be used as a basis for discussion its validity must be examined.

The above estimate of the absolute amount of nucleolar DNA is based upon a 2C value for mouse liver nuclei of 6·5 μg DNA. The most commonly quoted value for mouse is that of Vendrely (1955) who found 5 μg of DNA per diploid nucleus. Contemporary opinion, however, seems to favour a value for mouse nearer to that for rat, which is said to be between 6·4 and 6·9 μg per diploid nucleus (Thomson, Heagy, Hutchison & Davidson, 1953). Because of this situation I compared micro-photometrically tetraploid Feulgen-stained liver nuclei from rat and mouse. I found the FDC for mouse to be 42·1 ± 2·3 (20 nuclei measured), and that for rat to be 41·1 ± 3·2 (20 nuclei measured). These values indicate little more than a 5% difference between the two animals. Accordingly, I have taken as standard a mouse 2C value of 6·5 μg.
Probably the most important question in assessing the validity of the nucleolar DNA estimate is whether or not the nuclei measured had reached their final maximal DNA content. This question cannot be answered with certainty, since nuclei of more than 30 μ diameter were unmeasurable. However, the pattern of build-up of nuclear DNA is worth reconsidering in this context.

While the chromatin cap is visible and compact the FDC of an oocyte nucleus increases from about 40 units to about 90 units. As the cap disperses and the nucleus grows to a diameter of about 25 μ the FDC value increases further to about 135 units. FDC values for nuclei larger than 25 μ diameter range from 135 to 160, and these values are apparently unrelated to nuclear size. On the basis of these observations I suggest that DNA synthesis is confined to nuclei in the cap and immediate post-cap stages and that nuclei of more than 25 μ diameter in which no cap is distinguishable have the final maximal amount of DNA. This suggestion is supported by the observation that in autoradiographs of ovaries incubated in the presence of [3H]thymidine, cap nuclei are labelled, and large post-cap nuclei are unlabelled.

A yolky oocyte nucleus from *Xenopus* contains 500–1500 nucleoli. These nucleoli can be isolated in such a way as to destroy the nucleolar cortices and reduce the nucleolar cores to small granular rings (Macgregor, 1965, 1967; Miller, 1966). Most nucleoli from *Xenopus* oocytes have more than one core. Some have as many as eight. The average number of cores per oocyte nucleus has been estimated by Perkowska (Perkowska, Macgregor & Birnstiel, 1967) as 5265 ± 145 (10 nuclei counted). Birnstiel et al. (1966) and Wallace & Birnstiel (1966) have found that in *Xenopus* somatic tissues the 28S and 18S ribosomal DNA complements together constitute 0-13–0-22 % of the total cellular DNA (6 μg). If one supposes the DNA of one nucleolar ring in an oocyte to be a replica of the nucleolar organizer then such a ring may be expected to contain 0-065–0-11 % of 6 μg DNA and 5200 such rings would together contain 335–557 % of 6 μg DNA, or 20-2–33-5 μg DNA. On the basis of the above estimates we have (Perkowska et al. 1968) suggested that each nucleolar DNA ring in a yolky *Xenopus* oocyte is a complete replica of the *Xenopus* nucleolar organizer.

The fact that early cap nuclei are less heavily labelled with [3H]thymidine than later cap nuclei provides a clue as to the mechanism of replication of nucleolar DNA. Miller (1966) has suggested two alternative (but not mutually exclusive) ways in which the chromosomal nucleolar organizer may give rise to the many detached nucleolar organizers of an amphibian oocyte: (1) the nucleolar organizers on the chromosomes are repetitively duplicated and the copies detached; or (2) one or more single copies are duplicated off the chromosomal nucleolar organizer and detached, and these detached copies undergo a further series of duplications to produce the final number of replicas. The observations on labelling of cap nuclei support the second alternative.

Nuclei in late stages of nucleolar DNA synthesis have more replicating sites, and therefore incorporate more thymidine in a given time, than nuclei in earlier stages. Were the chromosomal nucleolar organizers the only sites of replication then nuclei in all stages of cap formation would have been similarly labelled.

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REFERENCES


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Figs. 1–9 were all photographed from one Feulgen-stained squash preparation of a single *Xenopus* ovary. The scale on Fig. 1 applies also to Figs. 2–6, that on Fig. 7 applies also to Figs. 8 and 9.

Fig. 1. Oogonial nucleus.

Fig. 2. Zygotene nuclei.

Fig. 3. Pachytene nucleus with a thin cap of Feulgen-positive material around its uppermost edge (arrowed).

Fig. 4. Pachytene nucleus with a conspicuous cap of extrachromosomal DNA (arrowed).

Fig. 5. Later pachytene nucleus. This nucleus had one of the largest condensed caps of DNA found in the preparation. The ends of the chromosomes can be seen near the lower edge of the nucleus, pointing away from the cap.

Fig. 6. Early post-pachytene nucleus in which the DNA cap has started to disperse. The cap at this stage has a faintly spotty appearance.

Fig. 7. Post-pachytene nucleus in which the DNA cap has almost completely dispersed into small Feulgen-positive granules.

Fig. 8. Diffuse diplotene nucleus. The extrachromosomal DNA has completely dispersed and the nucleus has a more or less homogeneous appearance.

Fig. 9. Diffuse diplotene nucleus. This nucleus was one of the largest in the preparation. It is in nuclei of about this stage that the chromosomes may begin to assume the lampbrush form. The extrachromosomal DNA is in the form of tiny granules and is no longer distinguishable from the chromosomal material.

Figs. 10–13. Autoradiographs of nuclei from an ovary which was incubated for 6 h with [*H*]thymidine. All the nuclei were photographed from the same preparation.

Fig. 10. Autoradiograph of an early pachytene nucleus with a small cap of extrachromosomal DNA. There are about 50 silver grains concentrated over the DNA cap.

Fig. 11. Autoradiograph of a later pachytene nucleus; 100–150 silver grains overlie the DNA cap.

Fig. 12. Autoradiograph of a late pachytene nucleus in which the DNA cap has started to disperse; 150–200 silver grains overlie the DNA cap.

Fig. 13. A post-pachytene nucleus with a well dispersed cap. There are no silver grains over this nucleus.
Figs. 14–21. Photographs of Feulgen-stained nuclei from one squash preparation of a *Xenopus* ovary. The nuclei are in various stages of meiosis from zygotene (Fig. 14) through pachytene (Figs. 15, 16), post-pachytene (Figs. 17, 18), and diffuse diplotene (Figs. 19–21). The numbers to the right of each nucleus represent the Feulgen dye content values of the nuclei. This preparation was typical in that post-pachytene nuclei in which the DNA cap had more or less dispersed had as much DNA as much larger diffuse diplotene nuclei. The corresponding value for 8C mouse liver nuclei on the same slide was 81.

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